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An increased ratio of fungi to bacteria indicates greater potential for N_2O production in a grazed grassland exposed to elevated CO_2

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ABSTRACT

Nitrous oxide (N_2O) is an important greenhouse gas and emissions of N_2O have been shown to increase under elevated CO_2 (eCO_2) resulting in a positive feedback on climate change. CO_2-driven increases under grassland have often been associated with greater N_2O emitted during denitrification. We examined the soils from a Free Air Carbon Dioxide Enrichment (FACE) experiment on grassland on the west coast of the North Island of New Zealand that had received long-term exposure to elevated CO_2. Importantly, the grassland was grazed thus representing much of the world's grassland situation and providing data for a land use that has not been well studied. We conducted soil incubations where a fungicide and bactericide were used to isolate the contribution of bacteria and fungi to potential N_2O production using denitrification enzyme activity (DEA). We found greater gene abundance of fungi under eCO_2 and reduced bacterial gene abundance. N_2O DEA was dominated by fungi in both ambient and elevated CO_2. Total potential N_2O emissions were 49% higher under eCO_2 entirely due to greater emissions from the fungal component. An increasing fungal contribution to N_2O emissions presents a challenge to mitigation as, to date, mitigations have largely been targeted at bacteria.

1. Introduction

Nitrous oxide (N_2O) is the third most important anthropogenic greenhouse gas and has now become the most damaging stratospheric ozone depleting substrate making it a major form of nitrogen pollution (Davidson and Kanter, 2014). Any change in N_2O emissions in response to climate change drivers are important as this could result in a positive or negative feedback on greenhouse gas forcing (van Groenigen et al., 2011).

Grasslands cover 37% of the ice-free surface of the globe (O'Mara, 2012) and experimental evidence suggests that they will produce an increasing amount of N_2O as the CO_2 concentration rises (Baggs et al., 2003b; Kammann et al., 2008; Cantarel et al., 2012). The importance of denitrification in this CO_2 effect on grasslands has been consistently identified. For example, Cantarel et al. (2012), in an upland grassland, and Baggs et al. (2003a) in a heavily fertilized lowland grassland, saw increased N_2O produced from denitrification in response to elevated CO_2 (eCO_2). Regan et al. (2011) reported higher N_2O emissions under eCO_2 in a German meadow after 10 years of CO_2 enrichment and suggested these increased N_2O emissions might be caused by a higher proportion of N_2O producing rather than N_2O consuming denitrifiers at eCO_2.

Studies of denitrification have focused largely on bacterial activity although fungal denitrification has been shown to be very active in grassland soils (Laughlin and Stevens, 2002). A fungal component to denitrification would be important for N_2O production as fungi generally lack an N_2O reductase and so the gaseous emission is N_2O rather than N_2 (Baggs, 2011). As one of the most common responses to eCO_2 is an increasing fungal:bacterial microbial biomass ratio in the soil (Rillig et al., 1999; Kandeler et al., 2008; Guenet et al., 2012; Hayden et al., 2012) resulting from greater carbon (C) inputs to the rhizosphere (Rillig et al., 1999; Drigo and Kowalchuk, 2013) we might expect that a greater proportion of soil processes could be catalyzed by fungi under eCO_2 including, perhaps, N_2O production. Nitrous oxide production by fungi has long been identified in grasslands (Laughlin and Stevens, 2002) and has now become widely recognized as a component of N_2O production that was previously thought to be dominated by bacteria (Chen et al., 2014; Maeda et al., 2015).

In this paper we examined soil samples from a long-running Free Air Carbon Dioxide Enrichment (FACE) experiment on grassland grazed by

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sheep (Newton et al., 2014) and asked a) is there potential for higher N_2O emissions under eCO_2 and b) if so, does a change in the fungal contribution to denitrification play a role? We were encouraged to examine these questions by previous studies on this experiment that have suggested higher N_2O emissions under eCO_2 related to a fungal source (Rütting et al., 2010) and a reduction in the activity of N_2O reductase (expression of *nosZ* genes) in eCO_2 soil (Zhong et al., 2015) that could result in greater N_2O relative to N_2 production from denitrification.

2. Materials and methods

2.1. The New Zealand FACE experiment

Soil was collected from the New Zealand FACE experiment which is located at 40°14′S, and 175°16′ E where the long-term (since 1945) mean annual temperature was 12.9 °C and annual rainfall 870 mm. The experiment was established on a pasture that had not been resown for several decades and contained a mix of C3 and C4 grasses, legumes and forbs (Edwards et al., 2001a). The soil was a Mollic Psammaquent – soil properties and changes in soil properties over time can be found in Ross et al. (2013). Fertiliser (phosphorus, potassium and sulfur) was added (Newton et al., 2010) but not nitrogen (N) fertiliser – the N input coming from nitrogen fixation by legumes which is typical of low input systems in temperate regions (Lüscher et al., 2014). The experiment had six 12 m diameter areas described as rings. Three of the rings were enriched with CO_2 using FACE technology (Edwards et al., 2001b) and three were left at ambient CO_2.

Enrichment started in October 1997 and was continuous for each day of the year until December 2011 with a target concentration of 475 ppm CO_2; there was then a hiatus while the facility was refurbished and enrichment recommenced in October 2013 with a target concentration of 500 ppm. The rings were irrigated occasionally during the summer period (December-February) post-2013. The rings were periodically grazed by adult sheep, details of the grazing protocol and actual grazing times can be found in Newton et al. (2014).

2.2. Soil sampling

Soil samples were collected from each ring on 11 November 2013 (one month after CO_2 enrichment restarted) and 13 March 2015. Twenty soil cores were collected in each ring to a depth of 75 mm using a 25 mm diameter soil corer and were then bulked. Fresh composite samples were subsampled for soil moisture determination, pH and inorganic N content (see Zhong et al., 2015 for methods) and denitrification potential, and a further subsample stored at −80 °C for DNA analysis.

2.3. Denitrification and N_2O

Denitrification potential was measured from denitrification enzyme activity (DEA). We conducted a soil incubation according to the protocol described by Patra et al. (2006) and Marusenko et al. (2013). Three sub-samples (12 g dry soil equivalent) from each soil sample were placed into 240 ml specimen bottles, then 7 ml of solution containing KNO_3 (50 μg NO_3^-N g^{-1} dry soil), glucose (0.5 mg C g^{-1} dry soil) and glutamic acid (0.5 mg C g^{-1} dry soil) were added. Additional distilled water was provided to achieve 100% saturation and therefore ideal conditions for denitrification. Three treatments were applied: (I) cycloheximide ($C_{15}H_{23}NO_4$; a fungicide) at 1.5 mg g^{-1} in solution, (II) streptomycin sulphate ($C_{42}H_{84}N_{14}O_{36}S_3$; a bactericide) at 3.0 mg g^{-1} in solution (Castaldi and Smith, 1998; Laughlin and Stevens, 2002), and (III) a no-inhibitor control. Note that archaea were not targeted in these treatments.

The headspace air of the bottles was replaced by nitrogen gas to provide anaerobic conditions and C_2H_2 (10% v/v) was added to inhibit N_2O reductase activity. The bottles were then sealed with a lid containing a rubber septum for gas sample collection and incubated at 28 °C for 48 h with constant agitation (180 rpm) in an orbital shaker (Lab-Line 3527; Boston, USA). During incubation, 12 ml gas samples were taken at 0, 24 and 48 h by syringe and injected into pre-evacuated 6 ml glass vials. The N_2O concentration of the gas samples was analysed by gas chromatography (Kelliher et al., 2012) at the National Centre for Nitrous Oxide Measurements, Lincoln University, Christchurch, New Zealand. The N_2O produced was calculated from a linear regression of the three sampling times (0, 24 and 48 h). An alternative approach using only the 0 and 48 h samplings gave very similar results indicating the effects of any non-linearity were minor.

Gas samples collected from bottles incubated with C_2H_2 were assumed to represent total denitrification activity ($N_2O + N_2$) and those without C_2H_2 the N_2O fraction only. The difference in N_2O concentration between the two samples was then the N_2 production potential. Treatment (I) (the fungicide treatment) was assumed to give values for bacterial denitrification enzyme activity (BDEA); treatment (II) (the bactericide treatment) to give the fungal component (FDEA) and treatment (III) (the no-inhibitor treatment) to give total denitrification enzyme activity (TDEA).

2.4. Gene abundance

Soil DNA was extracted from 0.5 g of the previously frozen and stored soil using a FastDNA™ Kit for Soil (QBIOgene, Irvine, CA, USA) following the manufacturer's instructions and stored at −20 °C until required for further analysis.

The abundance of bacteria and fungi was quantified in triplicate by real-time PCR using a LightCycler™ 480 II (Roche, Vienna, Austria). The real time PCR mixture contained 5 ng of soil DNA, 2 pmol of primers and 10 × SYBR Green iCycler iQ mixture (Roche Applied Science, Mannheim, Germany) in a total of 20 μl reaction volume. The primer details for bacteria were 341F 5′- CCT ACG GGA GGC AGC AG −3′ and 534R 5′- ATT ACC GCG GCT GCT GGC A −3′ (López-Gutiérrez et al., 2004) and the thermal cycle conditions were steps of 10 min at 95 °C; 35 cycles of PCR were performed as follows: 20 s at 95 °C, 15 s at 55 °C and 30 s at 72 °C. A final 5-min extension step completed the protocol. The primer details for fungi were FU18S1 5′-GGAAACTCACCAGGTCC AGA- 3′ derived from Nu-SSU-1196 and Nu-SSU-1536 5′-ATTGCAA TGCYCTATCCCCA-3′ (Borneman and Hartin, 2000) and the thermal cycle conditions were steps of 10 min at 95 °C; there were 40 cycles of PCR: 20 s at 95 °C, 30 s at 62 °C and 30 s at 72 °C with a final 5-min extension step.

2.5. Statistical analysis

The effects of CO_2 treatment and time were analysed by a mixed effects model (CO_2*time + (1|block/ring)) where CO_2, time and their interaction were fixed effects and ring nested within block was the random effect. To account for the small sample size (a total of 12 observations) a permutation test was used to obtain P-values using 999 iterations for each fixed term in the above model. The method is an extension of permutation tests for random effects in linear mixed models (Lee and Braun, 2012). The analysis was conducted using R version 3.4.1 packages 'lme4' and 'predictmeans' (R Core Writing Team, 2017).

3. Results

Gravimetric soil moisture was lower at the second sampling but not different between CO_2 treatments (P = 0.234) (ambient 29.1% (s.e.m. 1.47), eCO_2 30.5% (1.38) at the first sampling; ambient 13.3% (1.14), eCO_2 16.9% (1.55) at the second sampling). We measured pH at the second sampling and this was the same for both treatments (ambient 6.3 (s.e.m. 0.03), eCO_2 6.3 (0.02)). There was no CO_2 effect on soil

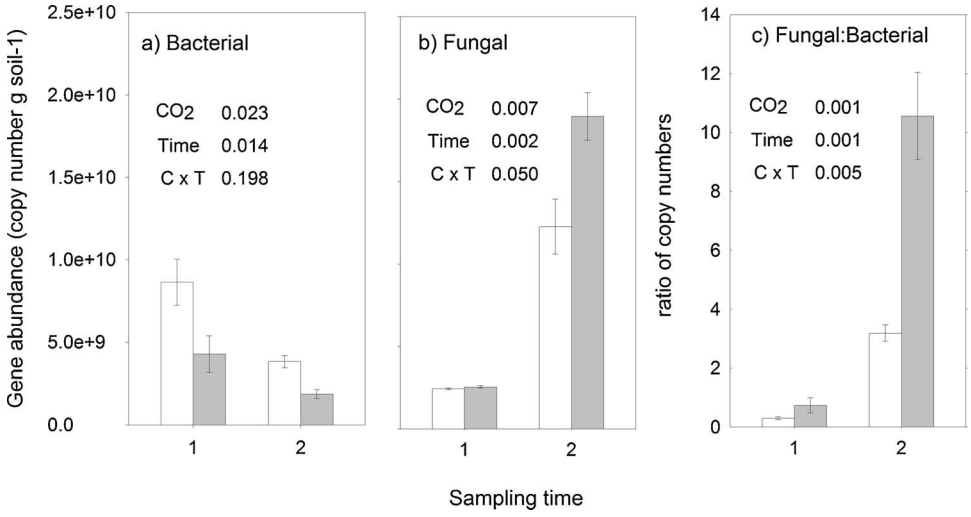

Fig 1. Gene abundance of a) bacteria and b) fungi and c) their ratio from soil sampled from ambient (open bars) and elevated CO_2 (filled bars) at two sampling times.

nitrate at either sampling time (p = 0.725 for CO_2 and p = 0.651 for CO_2 x Time) – ambient 7.3 mg kg dry soil^{-1} (s.e.m. 0.81), eCO_2 7.3 (0.84) at the first sampling; ambient 3.7 (0.52), eCO_2 4.4 (0.57) at the second sampling.

Bacterial gene abundance was significantly lower in eCO_2 (P = 0.023) and lower in both treatments at the second sampling (P = 0.014) (Fig. 1a). By contrast, there was a highly significant (P = 0.007) increase in fungal gene copy number at the second sampling (Fig. 1b) leading to a markedly greater fungal:bacterial ratio (Fig. 1c).

Total soil DEA_{N2O} was dominated by fungal activity in both ambient and eCO_2 (Fig. 2). Total soil DEA_{N2O} was greater in eCO_2 (P = 0.057) and higher at the first sampling (P = 0.003, Fig. 2a). The bacterial contribution to the N_2O flux was unchanged by eCO_2 and did not change over time (Fig. 2b). The fungal contribution was significantly higher under eCO_2 at both sampling times (P = 0.027, Fig. 2c). At both ambient and eCO_2, soil fungal DEA_{N2O} was lower at the second sampling despite fungal gene abundance being higher at this time (Fig. 1b).

There was no significant effect of CO_2 or time on DEA_{N2} (Fig. 3a). $BDEA_{N2}$ was higher at the second sampling (P = 0.011) but unaffected by eCO_2 (P = 0.129, Fig. 3b). No fungal N_2 was measured. The ratio of N_2:N_2O showed a CO_2 x time interaction (P = 0.030) such that the proportion of N_2O released was significantly greater under eCO_2 at the second sampling by comparison with the ambient treatment (Fig. 4).

4. Discussion

Fungi contributed 86% of the N_2O production potential in ambient and 95% in eCO_2 thus supporting a growing literature on the importance of fungi in N_2O emissions (Mothapo et al., 2013; Chen et al., 2014; Maeda et al., 2015). Our estimate of the fungal contribution is comparable to the 89% fungal contribution measured by Laughlin and Stevens (2002) from temperate grasslands soils using a similar method to ours but is greater than the 40–51% fungal contribution observed across different ecosystems by Chen et al. (2014).

We found increased N_2O production potential from denitrification in soils exposed to eCO_2, supporting the results of other grassland experiments (Baggs et al., 2003a; Regan et al., 2011; Cantarel et al., 2012). This could not be ascribed to changes in soil nitrate concentrations as these were not different between the CO_2 treatments. The new information here is that the 49% increase in potential N_2O emissions under eCO_2 was entirely due to fungal activity. It should be noted that this increase was not simply a result of greater fungal abundance as there was a higher $FDEA_{N2O}$ at the first sampling when fungal gene copy numbers were lower. We assume, as the incubation methods gave standard conditions, that the difference in fungal production could

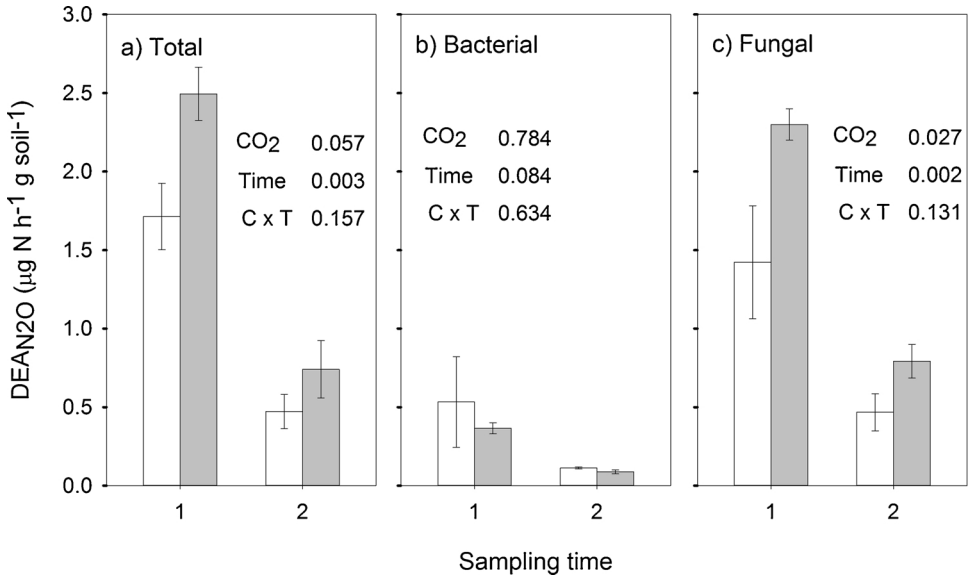

Fig. 2. The N_2O produced from a) total denitrifying enzyme activity (DEA) b) bacterial DEA and c) fungal DEA measured during incubation of soil sampled from ambient (open bars) and elevated CO_2 (filled bars) at two sampling times.

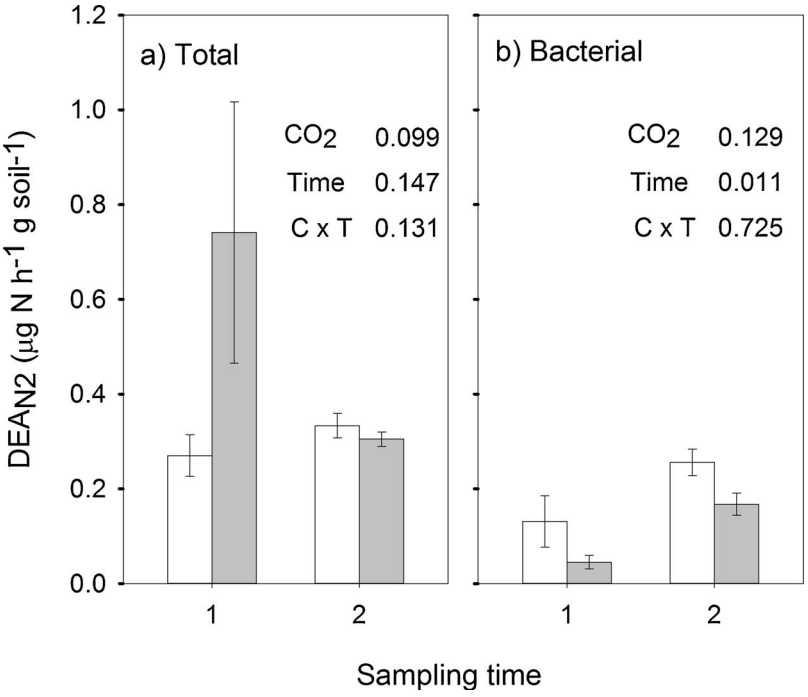

Fig. 3. The N_2 produced from a) total denitrifying enzyme activity (DEA) b) bacterial DEA during incubation of soil sampled from ambient (open bars) and elevated CO_2 (filled bars) at two sampling times.

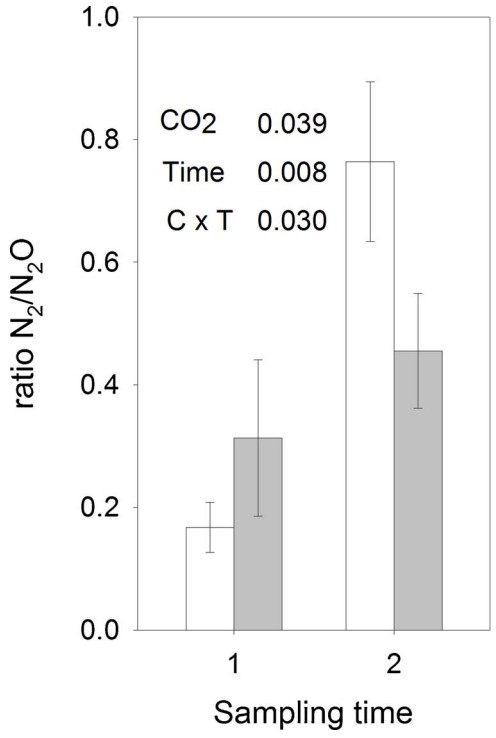

Fig. 4. The ratio of N_2/N_2O produced by denitrification during incubation of soil sampled from ambient (open bars) and elevated CO_2 (filled bars) at two sampling times.

relate to differences in fungal community structure perhaps as a consequence of the different length of time since CO_2 enrichment re-started. Our results are consistent with an increase in a heterotrophic contribution to N_2O production inferred from a ^{15}N tracer study on this same experiment (Rütting et al., 2010) after 10 years of continuous enrichment and the previous study of Zhong et al. (2015) identifying greater potential for N_2O production and greater heterotrophic activity under eCO_2 in the same soil.

We are not aware of other eCO_2 studies where an increasing fungal contribution to N_2O has been shown but can assume that further investigation would find other examples as an increasing fungal abundance is commonly observed in response to eCO_2 (Rillig et al., 1999; Kandeler et al., 2008; Guenet et al., 2012; Hayden et al., 2012). In our experiment a higher fungal:bacterial ratio was already evident at the first sampling (one month after the CO_2 enrichment re-started) either indicating a very rapid response to CO_2, or, more likely, a consequence of the previous 14 years of CO_2 enrichment. In a study of shorter term CO_2 enrichment (5 years) Kandeler et al. (2008) found cessation of the enrichment led to a shift in the abundance of bacteria and fungi back towards the control value, commenting that the 5 years of enrichment had not been sufficient time to shift the available substrates to the point where the increased fungal abundance was stable.

The increase in the ratio of fungi to bacteria from the first to the second sampling is likely a result of the re-institution of grazing after this was suspended during the refitting of the experiment as grazing is known to favour fungal over bacterial abundance (Bardgett et al., 1997). The mechanism of change in the microbial populations almost certainly relates to an increased input of C to the soil under eCO_2, particularly in C to the rhizosphere (Drigo et al., 2008; Drigo and Kowalchuk, 2013); we have previously seen in this experiment a greater C exudation from roots under eCO_2 (Allard et al., 2006) and greater extractable soil C (the most labile C fraction in the soil) (Ross et al., 2013). A relationship between increased fungal N_2O production under an enhanced organic C supply has clearly been shown by Laughlin and Stevens (2002) and was associated with a stimulation of heterotrophic nitrification.

Our methodology does not exclude a role for archaea in denitrification and we cannot estimate their contribution. Previous research on grasslands has focussed on fungal and bacterial denitrification e.g. Saggar et al. (2013) because N_2O production by archaea in soil has not been proven (Butterbach-Bahl et al., 2013). However, archaea are widespread in soils, are involved in denitrification (Cabello et al., 2004) and may yet be shown to play an important part in soil N_2O emissions (Butterbach-Bahl et al., 2013). It would be an improvement in experimental design if future experiments of this kind included a treatment where both bacteria and fungi were inhibited in order to quantify the archaeal component.

The total gaseous loss of N from the soil (due to denitrification) is the sum of N_2O and N_2. Baggs et al. (2003a) found a higher ratio of N_2/

N_2O under eCO_2 while we found a reduced ratio. This difference may be explained by an increased contribution from fungi in our experiment (and thus lower production of N_2) whereas in the Bragg et al. (2003a) experiment there was no change in the fungal:bacterial ratio (Drissner et al., 2007) perhaps because of the high level of N fertiliser applied that would tend to favour a higher bacterial abundance (Bardgett et al., 1999). Our results were more similar to a lower-fertility grassland FACE experiment in Germany where denitrification was the main source of N_2O emissions and where there was a much lower incidence of *nosZ* genes at elevated CO_2 meaning a lower capacity to reduce N_2O to N_2 (Regan et al., 2011).

Interestingly, in our experiment, the total N_2 produced was greater than the bacterial N_2 produced although there was no fungal contribution (Fig. 3). This may be evidence of abiotic production of hybrid N_2 (Phillips et al., 2016) possibly due to artefacts of the incubation assay we used (i.e. accumulation of NO_2 due to repression of NO_2 oxidation by antibiotics).

5. Conclusions

The positive feedback on atmospheric greenhouse gas concentrations through a possible CO_2-stimulated increase in N_2O emissions (of fungal origin) is of concern on two fronts. First, because grazed grassland is a very significant land use by area (37%, (O'Mara, 2012)) and thus a change in emissions from this ecosystem assumes major importance. Second, because current mitigation technologies or practices, such as nitrification inhibitors, natural (Subbarao et al., 2015) or synthetic (Di et al., 2010), are directed at bacterial rather than fungal N_2O production. In addition, in contrast to bacterial denitrification, fungal denitrification generally lacks the N_2O reduction pathway resulting in greater N_2O emissions (Baggs, 2011). How an increasing rate of emissions from grassland might be managed and whether a CO_2-stimulated fungal pathway is relevant to other systems are clearly important questions for the future.

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References

Allard, V., Robin, C., Newton, P.C.D., Lieffering, M., Soussana, J.F., 2006. Short and long-term effects of elevated CO_2 on *Lolium perenne* rhizodeposition and its consequences on soil organic matter turnover and plant N yield. Soil Biol. Biochem. 38, 1178–1187.

Baggs, E.M., Richter, M., Cadisch, G., Hartwig, U.A., 2003a. Denitrification in grass swards is increased under elevated atmospheric CO_2. Soil Biol. Biochem. 35, 729–732.

Baggs, E.M., Richter, M., Hartwig, U.A., Cadisch, G., 2003b. Nitrous oxide emissions from grass swards during the eighth year of elevated atmospheric pCO_2 (Swiss FACE). Glob. Change Biol. 9, 1214–1222.

Baggs, E.M., 2011. Soil microbial sources of nitrous oxide: recent advances in knowledge, emerging challenges and future direction. Curr. Opin. Environ. Sustain. 3, 321–327.

Bardgett, R.D., Leemans, D.K., Cook, R., Hobbs, P.J., 1997. Seasonality of the soil biota of grazed and ungrazed hill grasslands. Soil Biol. Biochem. 29, 1285–1294.

Bardgett, R.D., Mawdsley, J.L., Edwards, S., Hobbs, P.J., Rodwell, J.S., Davies, W.J., 1999. Plant species and nitrogen effects on soil biological properties of temperate upland grasslands. Funct. Ecol. 13, 650–660.

Borneman, J., Hartin, R.J., 2000. PCR primers that amplify fungal rRNA genes from environmental samples. Appl. Environ. Microbiol. 66, 4356–4360.

Butterbach-Bahl, K., Baggs, E.M., Dannenmann, M., Kiese, R., Zechmeister-Boltenstern, S., 2013. Nitrous oxide emissions from soils: how well do we understand the processes and their controls? Phil. Trans. R. Soc. B : Biol. Sci. 368.

Cabello, P., Roldán, M.D., Moreno-Vivián, C., 2004. Nitrate reduction and the nitrogen cycle in archaea. Microbiology 150 (11), 3527–3546.

Cantarel, A.A.M., Bloor, J.M.G., Pommier, T., Guillaumaud, N., Moirot, C., Soussana, J.F., Poly, F., 2012. Four years of experimental climate change modifies the microbial drivers of N 2O fluxes in an upland grassland ecosystem. Glob. Change Biol. 18, 2520–2531.

Castaldi, S., Smith, K.A., 1998. Effect of cycloheximide on N_2O and NO_3?production in a forest and an agricultural soil. Biol. Fert. Soils 27, 27–34.

Chen, H., Mothapo, N.V., Shi, W., 2014. The significant contribution of fungi to soil N_2O production across diverse ecosystems. Appl. Soil Ecol. 73, 70–77.

Davidson, E.A., Kanter, D., 2014. Inventories and scenarios of nitrous oxide emissions. Environ. Res. Lett. 9, 105012.

Di, H.J., Cameron, K.C., Sherlock, R.R., Shen, J.-P., He, J.-Z., Winefield, C.S., 2010. Nitrous oxide emissions from grazed grassland as affected by a nitrification inhibitor, dicyandiamide, and relationships with ammonia-oxidizing bacteria and archaea. J. Soils Sediments 10, 943–954.

Drigo, B., Kowalchuk, G.A., 2013. Rhizosphere responses to elevated CO_2. Molecular Microbial Ecology of the Rhizosphere. John Wiley & Sons, Inc., pp. 1063–1074.

Drigo, B., Kowalchuk, G.A., Van Veen, J.A., 2008. Climate change goes underground: effects of elevated atmospheric CO_2 on microbial community structure and activities in the rhizosphere. Biol. Fert. Soils 44, 667–679.

Drissner, D., Blum, H., Tscherko, D., Kandeler, E., 2007. Nine years of enriched CO_2 changes the function and structural diversity of soil microorganisms in a grassland. Eur. J. Soil Sci. 58, 260–269.

Edwards, G., Clark, H., Newton, P.C.D., 2001a. The effects of elevated CO_2 on seed production and seedling recruitment in a sheep-grazed pasture. Oecologia 127, 383–394.

Edwards, G.R., Newton, P.C.D., Tilbrook, J.C., Clark, H., 2001b. Seedling performance of pasture species under elevated CO_2. New Phytologist 150, 359–369.

Guenet, B., Lenhart, K., Leloup, J., Giusti-Miller, S., Pouteau, V., Mora, P., Nunan, N., Abbadie, L., 2012. The impact of long-term CO_2 enrichment and moisture levels on soil microbial community structure and enzyme activities. Geoderma 170, 331–336.

Hayden, H.L., Mele, P.M., Bougoure, D.S., Allan, C.Y., Norng, S., Piceno, Y.M., Brodie, E.L., Desantis, T.Z., Andersen, G.L., Williams, A.L., Hovenden, M.J., 2012. Changes in the microbial community structure of bacteria, archaea and fungi in response to elevated CO_2 and warming in an Australian native grassland soil. Environ. Microbiol. 14, 3081–3096.

Kammann, C., Müller, C., Grünhage, L., Jäger, H.J., 2008. Elevated CO_2 stimulates N_2O emissions in permanent grassland. Soil Biol. Biochem. 40, 2194–2205.

Kandeler, E., Mosier, A.R., Morgan, J.A., Milchunas, D.G., King, J.Y., Rudolph, S., Tscherko, D., 2008. Transient elevation of carbon dioxide modifies the microbial community composition in a semi-arid grassland. Soil Biol. Biochem. 40, 162–171.

Kelliher, F.M., Clough, T.J., Premaratne, M., Laughlin, R.J., McGeough, K.L., Harvey, M., McMillan, A.M.S., Reid, A., Saggar, S., 2012. Air sample collection, storage and analysis. In: De Klein, C.A.M., Harvey, M. (Eds.), Nitrous Oxide Chamber Methodology Guidelines. New Zealand Ministry for Primary Industries, Wellington, New Zealand, pp. 56–72.

López-Gutiérrez, J.C., Henry, S., Hallet, S., Martin-Laurent, F., Catroux, G., Philippot, L., 2004. Quantification of a novel group of nitrate-reducing bacteria in the environment by real-time PCR. J. Microbiol. Methods 57, 399–407.

Lüscher, A., Mueller-Harvey, I., Soussana, J.F., Rees, R.M., Peyraud, J.L., 2014. Potential of legume-based grassland–livestock systems in Europe: a review. Grass Forage Sci. 69, 206–228.

Laughlin, R.J., Stevens, R.J., 2002. Evidence for fungal dominance of denitrification and codenitrification in a grassland soil. Soil Sci. Soc. Am. J. 66, 1540–1548.

Lee, O.E., Braun, T.M., 2012. Permutation tests for random effects in linear mixed models. Biometrics 68, 486–493.

Maeda, K., Spor, A., Edel-Hermann, V., Heraud, C., Breuil, M.-C., Bizouard, F., Toyoda, S., Yoshida, N., Steinberg, C., Philippot, L., 2015. N_2O production, a widespread trait in fungi. Sci. Rep. 5, 9697.

Marusenko, Y., Huber, D.P., Hall, S.J., 2013. Fungi mediate nitrous oxide production but not ammonia oxidation in aridland soils of the southwestern US. Soil Biol. Biochem. 63, 24–36.

Mothapo, N.V., Chen, H., Cubeta, M.A., Shi, W., 2013. Nitrous oxide producing activity of diverse fungi from distinct agroecosystems. Soil Biol. Biochem. 66, 94–101.

Newton, P.C.D., Lieffering, M., Bowatte, W.M.S.D., Brock, S.C., Hunt, C.L., Theobald, P.W., Ross, D.J., 2010. The rate of progression and stability of progressive nitrogen limitation at elevated atmospheric CO_2 in a grazed grassland over 11 years of Free Air CO_2 enrichment. Plant Soil 336, 433–441.

Newton, P.C.D., Lieffering, M., Parsons, A.J., Brock, S.C., Theobald, P.W., Hunt, C.L., Luo, D., Hovenden, M.J., 2014. Selective grazing modifies previously anticipated responses of plant community composition to elevated CO_2 in temperate grassland. Glob. Change Biol. 20, 158–169.

O'Mara, F.P., 2012. The role of grasslands in food security and climate change. Ann. Botany 110, 1263–1270.

Patra, A.K., Abbadie, L., Clays-Josserand, A., Degrange, V., Grayston, S.J., Guillaumaud, N., Loiseau, P., Louault, F., Mahmood, S., Nazaret, S., Philippot, L., Poly, F., Prosser, J.I., Roux, X.L., 2006. Effects of management regime and plant species on the enzyme activity and genetic structure of N-fixing, denitrifying and nitrifying bacterial communities in grassland soils. Environ. Microbiol. 8, 1005–1016.

Phillips, R.L., Song, B., McMillan, A.M., Grelet, G., Weir, B.S., Palmada, T., Tobias, C., 2016. Chemical formation of hybrid di-nitrogen calls fungal codenitrification into question. Sci. Rep. 6, 39077.

R Core Writing Team, 2017. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/.

Rütting, T., Clough, T.J., Muller, C., Lieffering, M., Newton, P.C.D., 2010. Ten years of elevated atmospheric carbon dioxide alters soil nitrogen transformations in a sheep-grazed pasture. Glob. Change Biol. 16, 2530–2542.

Regan, K., Kammann, C., Hartung, K., Lenhart, K., Müller, C., Philippot, L., Kandeler, E., Marhan, S., 2011. Can differences in microbial abundances help explain enhanced N 2O emissions in a permanent grassland under elevated atmospheric CO 2? Glob. Change Biol. 17, 3176–3186.

Rillig, M.C., Field, C.B., Allen, M.F., 1999. Soil biota responses to long-term atmospheric

CO_2 enrichment in two California annual grasslands. Oecologia 119, 572–577.
Ross, D.J., Newton, P.C.D., Tate, K.R., Luo, D., 2013. Impact of a low level of CO_2 enrichment on soil carbon and nitrogen pools and mineralization rates over ten years in a seasonally dry, grazed pasture. Soil Biol. Biochem. 58, 265–274.
Saggar, S., Jha, N., Deslippe, J., Bolan, N.S., Luo, J., Giltrap, D.L., Kim, D.G., Zaman, M., Tillman, R.W., 2013. Denitrification and N_2O:N_2 production in temperate grasslands: processes, measurements, modelling and mitigating negative impacts. Sci. Total Environ. 465 (Suppl. C), 173–195.
Subbarao, G.V., Yoshihashi, T., Worthington, M., Nakahara, K., Ando, Y., Sahrawat, K.L., Rao, I.M., Lata, J.-C., Kishii, M., Braun, H.-J., 2015. Suppression of soil nitrification by plants. Plant Sci. 233, 155–164.
Zhong, L., Bowatte, S., Newton, P.C.D., Hoogendoorn, C.J., Li, F.Y., Wang, Y., Luo, D., 2015. Soil N cycling processes in a pasture after the cessation of grazing and CO_2 enrichment. Geoderma 259-260, 62–70.
van Groenigen, K.J., Osenberg, C.W., Hungate, B.A., 2011. Increased soil emissions of potent greenhouse gases under increased atmospheric CO_2. Nature 475, 214–216.